RESEARCH

Occurrence of Condensed Tannins in Wheat and Feasibility for Reducing Pasture Bloat

Charles T. MacKown,* Brett F. Carver, and Jeffrey T. Edwards

ABSTRACT

Bloat can be a serious problem for ruminant livestock grazing pastures of winter wheat (*Triticum aestivum* L.) in the southern Great Plains. Tannins in forage can reduce the incidence and severity of bloat. We measured the content and variability of total phenolics and condensed tannins (CT) in forage extracts from wheat varieties and experimental lines (ExpLines) to assess the feasibility of developing improved varieties using conventional breeding methods to give producers a choice to reduce the incidence of bloat. Forage of 16 winter wheat varieties were collected in late fall at the start of grazing. Also, late fall forage samples and regrowth from late fall clipped forage and unclipped growth before jointing were collected for 221 diverse ExpLines and four Check varieties. Among the 16 adapted winter wheat varieties significant differences for the acid-butanol but not the vanillin CT assay were observed. Among the ExpLines, significant differences in extractable total phenolics were detected (range for all samples collected, 8.9–31.5 mg tannic acid g^{-1} dry wt.) and for one of the three forage collections using each CT assay. While we observed differences in CT reactive substances among the ExpLines, even the greatest equivalent amounts detected (12 mg quebracho g^{-1} dry wt. and 0.67 mg epicatechin g^{-1} dry wt.) are unlikely sufficient to render the forage bloat-safe. Perhaps those experimental lines with the most abundant CT levels could be used in a selection program to increase the level of CT in wheat forage.

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Abbreviations: BT Exp, breeding trial experiment; CT, condensed tannin; epicatechin-eq CT, epicatechin-equivalent condensed tannin; DFR, dihydroflavanol reductase; ExpLines, experimental lines; MCW, methanol–chloroform–water; quebracho-eq CT, quebracho-equivalent condensed tannin; tannic acid-eq TP, tannic acid-equivalent total phenolics; VT Exp, variety trial experiment.

Readily consumed forages with high digestibility and abundant soluble proteins can cause pasture bloat in ruminants (Clarke and Reid, 1974; Majak et al., 2003). Even though winter wheat (*Triticum aestivum* L.) forage has these characteristics, it is the predominant cool-season forage in the southern Great Plains, where it is often grown as a dual-purpose crop (graze + grain) and is considered excellent forage, capable of producing stocker cattle (*Bos taurus* L.) weight gains greater than 1.4 kg d^{-1}. It has been estimated, however, that about 2 to 3% of stocker cattle that graze wheat pasture will die because of bloat (Horn et al., 1977).

More recently, a survey of Oklahoma farmers found that nearly 60% of the 2.5 million ha of Oklahoma wheat planted in the 1999–2000 season were grazed (Hossain et al., 2004). During that season, 41% of those surveyed reported problems of pasture bloat in cattle grazing wheat, with more than 40% of the

Published in Crop Sci. 48:2470–2480 (2008).
doi: 10.2135/cropsci2008.01.0020

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death losses attributed to bloat, which can arise rapidly and unexpectedly. Because of the large number of stockers that pass through Oklahoma and graze winter wheat pastures, the overall economic loss to Oklahoma farmers and other stocker producers in the southern Great Plains using wheat pastures is substantial. For a 270-kg calf valued at $720 in today's market (April 2007), stocker deaths due to bloat represent an $8.64 million yearly loss to Oklahoma livestock operations. While supplements (mainly surfactants and ionophores) can be used to reduce occurrence of frothy pasture bloat, these practices require active intervention by producers and lose effectiveness if these supplements are not consumed daily by cattle (Bartley et al., 1975; Grigsby, 1984; Branine and Gaylean, 1990; Paisley and Horn, 1998; Min et al., 2005a, 2005b).

Interest in condensed tannins has increased among nutritionists, physiologists, and plant breeders because of their antioxidant and protein-precipitating properties and their role in forage quality, plant defenses, and human health (Aerts et al., 1999; Booker, 1999; Dixon and Sumner, 2003; Dixon et al., 2005; Mueller-Harvey, 2006). Forages with moderate condensed tannin concentrations between 20 and 40 g kg^{-1} dry wt. have been proposed as suitable for bloat prevention (Barry et al., 1986; Aerts et al., 1999) and would reduce the cost of intervention practices for bloat prevention. Protein foams formed in the rumen are collapsed by condensed tannins in a dose-dependent process (Tanner et al., 1995). Moderate tannin levels offer additional benefits due to the precipitating reaction between tannins and soluble proteins in the rumen fluid. The interaction between tannins and protein (and their polypeptides) can increase forage protein utilization by enhancing passage of nitrogen from the rumen to the gut and decreasing nitrogen losses in the urine (Aerts et al., 1999; Barry and McNabb, 1999a and 1999b). Because of the diversity of hydrolyzable and condensed tannins, the optimum concentration of tannin required to elicit a beneficial effect yet avoid harmful effects associated with reduced forage intake and digestibility varies widely (Mueller-Harvey, 2006).

A better understanding of the expression and regulation of the many biochemical steps in the pathway of condensed tannins and other secondary metabolites has just begun. Considerable effort is being undertaken to manipulate the biochemical pathway regulating condensed tannins in alfalfa (*Medicago sativa* L.) and other legumes to make them bloat-safe, improve their nitrogen use by ruminants, and improve their feed value (Robbins et al., 1998; Marles et al., 2003; Robbins et al., 2003; Xie et al., 2003; Paolocci et al., 2007). In the leaves of wheat, researchers (Himi and Noda, 2004) recently demonstrated the presence of genes for DFR (dihydroflavanol reductase), an enzyme catalyzing an important step in the biosynthesis pathway upstream of anthocyanin and condensed-tannins formation. These DFRs differ in their promoter region and are expressed differentially depending on the plant organ. Expression of three Myb-type transcription factors (Tamyb 10-A1, Tamyb 10-B1, and Tamyb 10-D1 on chromosomes 3A, 3B, and 3D, respectively) is required for red seed coat in wheat and is weakly expressed in leaf tissue (Himi and Noda, 2005). Accumulation of condensed tannins in other wheat organs including leaves may also occur, but based on the incidence of bloat in cattle grazing today's wheat varieties, they are probably at a low level.

It may be possible, using various selection methods, to exploit the natural variability in leaf tannins and develop wheat varieties having elevated leaf tannins that would lessen the incidence of bloat and improve nitrogen utilization by ruminants grazing wheat pastures. As a first step, the extent of variation in tannin levels of wheat leaves must be determined. The objective of this research was to measure the variability of extractable total phenolics and condensed tannin fractions in wheat forage collected from adapted varieties and experimental lines with a broad genetic base and assess the feasibility of developing wheat with reduced bloat potential and possibly increasing the use of forage protein. In dual-purpose (graze + grain) wheat production systems, periods of lush pasture growth in the fall and late winter can coincide with incidences of pasture bloat. High wheat forage allowance resulted in greater nonlethal bloat severity than low forage allowance, with incidences of bloat occurring in at least 1 out of 26 steers in 66% of 70 observations made during a 9-wk period that included vegetative and reproductive stages of wheat growth (Min et al., 2005b). In our study, we chose to measure extractable phenolics in wheat forage at the onset of late fall grazing and just before jointing (pre–Feekes Stage 6; Large, 1954) in early March, a period corresponding to the grazing cycle of dual-purpose wheat.

MATERIALS AND METHODS

Varieties and Experimental Lines

Wheat plants were sampled from field experiments planted in 2004 and 2005. The first field experiment was a forage variety trial (VT Exp) and included 16 wheat entries located 35°22′42″ N, 97°51′35″ W, 390 m in Canadian County, Oklahoma, on a Pond Creek silt loam soil (fine-silty, mixed, thermic Pachic Argiustolls). Plots 6.1-m long with eight rows spaced 15-cm apart were sown at 134 kg ha^{-1} into a conventionally tilled seedbed on 20 Sept. 2004. Fertilizer (56 kg ha^{-1} of 18–46–0) was applied in the furrow at planting. Variety plots were arranged in a randomized complete block design with four replications. Forage samples at about Feekes Stage 4 were collected 10 Dec. 2004 from each plot by clipping to a height of ~4 cm to generate two random grab samples (~10-cm row length each). The samples were immediately frozen on dry ice, returned to the lab, and placed in an ultralow freezer (−78°C).

In the second experiment located 36°23′14″ N, 98°06′32″ W, 390 m in Garfield County at the Oklahoma State University

North Central Research Station, a breeding trial (BT Exp) included four cultivars ('Duster', 'Endurance', 'Jagger', and 'Overley') and 221 experimental lines (Supplementary Table S1) that were evaluated during the 2005–2006 crop year. The experimental design was an augmented randomized complete block consisting of the experimental lines distributed among 12 blocks and the four cultivars within each block. Plots 3.05-m long with five rows spaced 23-cm apart were sown 20 Sept. 2005 at 64 kg ha^{-1} into a conventionally tilled seedbed of a Grant silt loam soil (fine-silty, mixed, thermic Udic Argiustolls). Fertilizer was broadcast applied before planting according to soil-test recommendations to reach a 4030 kg ha^{-1} grain yield goal. Forage samples were collected late fall (29 Nov. 2005, about Feekes Growth Stage 4) and in late winter over a 3-d period (27 Feb. to 1 Mar. 2006, before Feekes Growth Stage 6). For the late fall forage collection, a 0.5-m length of an interior row was cut to a stubble height of ~4 cm, and the samples were frozen on dry ice, returned to the lab, and placed in an ultralow freezer (−78°C). The same plots were sampled again; from each plot, forage regrowth after the late fall sample and a previously unclipped sample were collected as described above, except it took three consecutive days instead of one to harvest all 12 blocks.

Sample Processing

Frozen samples were lyophilized, weighed, and then returned to the ultralow freezer and removed as needed for grinding in a cyclone mill to pass a 1.0 mm screen. Ground samples were returned to the ultralow freezer and removed as needed for tannin analysis.

Forage Phenolics Extraction and Analyses

The ground forage samples (0.75 g) were extracted in 50-mL capped tubes overnight on a rotary platform at 150 rpm using 30 mL of a methanol–chloroform–water (MCW) mixture (13:4:3, v:v:v of MCW). Tissue was centrifuged, and an aliquot was partitioned into methanol–water and chloroform phases by adding chloroform and water followed by centrifugation. The methanol–water phase was retained for total phenolics and condensed tannin functional group assays.

Total extractable phenolics were measured using a color-stabilized Prussian blue method and tannic acid as the standard (Graham, 1992). The method was adapted for a microplate reader and results were expressed as tannic acid– equivalent total phenolics (tannic acid–eq TP). In addition, the condensed tannin components of the total phenolics extraction were assayed by two methods. Aliquots of the methanol–water phase from the MCW extraction were dried in a vacuum centrifuge and stored in an ultralow freezer until assayed. For the acid-butanol condensed-tannin assay, the dried methanol–water aliquot was dissolved in 50% (v:v) methanol–water and measured using the method of Porter et al. (1986) and quebracho for a standard. Results were expressed as quebracho-equivalent condensed tannin (quebracho-eq CT). For the vanillin CT assay, the dried methanol–water aliquot was dissolved in an HCl–methanol mixture (8.34% v:v of HCl:methanol) and measured using the method of Makkar and Becker (1993). The method was adapted for a microplate reader and results were expressed as (–)-epicatechin-equivalent condensed tannins (epicatechin-eq CT).

Climate Data

Oklahoma Mesonet stations located 14.6 km (~9.1 mi) southwest of the VT Exp plots in Canadian County and about 0.4 km (0.25 mi) west of the BT Exp plots located in Garfield County provided measurements of environmental conditions. Summary data and daily environmental conditions preceding sampling were obtained from data maintained by the Oklahoma Climatological Survey (http://www.mesonet.org).

Data Analyses

All statistical analyses were performed using JMP 6 software (SAS Institute, 2006). The Fit Model Platform in JMP software was used for both experiments. The VT Exp data were analyzed using conventional ANOVA for a randomized complete block design. The protocol described by Scott and Milliken (1993) for the ANOVA of an augmented randomized complete block design was used for the BT Exp data. Each sample collection (late fall, late winter regrowth of late fall clipping, and late winter) was analyzed separately. These ANOVA produced two estimable effects associated with Checks (the four varieties Duster, Endurance, Jagger, and Overley) and ExpLines nested within Checks. Least-squares means were computed for all Checks and ExpLines using the nested classification in a mixed-model analysis, assuming block effects were random. The Checks source of variation provides a test of the null hypothesis that the four Check variety means and the mean of the ExpLines are equal. The ExpLines source of variation provides a test of the null hypothesis that all the experimental line means are equal. Multiple comparison tests of least-square means at $\alpha = 0.05$ used the Tukey Honestly Significant Difference command in JMP software. Univariate analysis was performed using the Distribution Platform of JMP software. Comparisons of a trait between sample collections in the BT Exp used the Matched Pairs Platform of JMP software.

RESULTS

Environmental Conditions

Fall precipitation between 1 Sept. and 10 Dec. 2004 near the VT Exp site was 47% above the 22.1 cm averaged across the same interval of the preceding 10 yr. Total solar radiation values for the 3 d before and on the day of sample collection in 2004 were not less than 71% of the possible maximum for daily total radiation, and daily average temperatures were consistently warmer than the long-term average (Table 1). In contrast, after planting the BT Exp in 2005, fall and winter precipitation was consistently below normal (Fig. 1). Total solar radiation at the BT Exp site ranged from 56 to 95% of the possible maximum daily total level and daily average air temperatures deviated above and below long-term values (Table 1).

Forage Traits for Variety Trial Experiment

The forage total extractable phenolics included condensed tannins and possibly other natural phenolic compounds (hydrolyzable tannins) that have protein-complexing properties. Among the 16 wheat varieties evaluated in 2004,

Table 1. Deviations from long-term (1994–2006) maximum possible daily total solar radiation and periodic average air temperature preceding the beginning and finish of forage sample collections from a forage variety trial experiment (VT Exp) located in Canadian County, Oklahoma, and a breeding trial experiment (BT Exp) located in Garfield County, Oklahoma.

Days from beginning of sample collection†	VT Exp	BT Exp	
	10 Dec. 2004	29 Nov. 2005	27 Feb.–1 Mar. 2006
	Solar radiation deviation (% of daily total maximum possible)‡		
–3	92	56	63
–2	71	75	74
–1	89	64	93
0	92	95	90
+1	–	–	93
+2	–	–	92
	Daily avg. temperature deviation (°C)§		
–3	2.5	5.7	2.3
–2	4.8	5.3	1.5
–1	4.3	–3.3	–1.5
0	3.0	–5.1	6.6
+1	–	–	10.2
+2	–	–	14.7

†Three consecutive days were required to make late winter sample collections of a previously unclipped sample and regrowth from the late fall sample collection.

‡Maximum possible daily total solar radiation values were 12.6 MJ m^{-2} for the VT Exp and 12.8 and 19.4 MJ m^{-2} for the late fall and late winter harvest dates of the BT Exp, respectively.

§Long-term average daily temperatures for the 4- or 6-d intervals were 4.1°C for the VT Exp and 7.5 and 6.7°C for the late fall and late winter harvest dates of the BT Exp, respectively.

the concentrations of total extractable phenolics expressed as tannic acid–eq TP were not significantly different ($P > 0.05$). The overall average tannic acid equivalent was 17.5 mg g^{-1} dry wt.; measured levels ranged from 15 to 20 mg g^{-1} dry wt. (Fig. 2). Extractable quebracho condensed tannin equivalents ranged from 2.5 to about 3.5 mg g^{-1} dry wt., with significant differences ($P \leq 0.05$) among the 16 varieties (Fig. 2). Only a few varieties differed from each other; for example, Jagger had a greater quebracho-eq CT concentration than Custer. Epicatechin-equivalent condensed tannins detected with the vanillin assay were not significantly different ($P > 0.05$) among the 16 varieties and averaged 0.47 mg g^{-1} dry wt (Fig. 2).

Forage Traits for Breeding Trial Experiment

Significant effects existed for both the Check and ExpLines (Checks) sources of variation (Table 2). A significant Check effect across all forage sample collections occurred for extractable tannic acid equivalent, while one or more of the sample collections for quebracho-eq CT concentration had a significant Check effect. For each phenolic acid fraction assayed, at least one of the sample collections exhibited a significant difference ($P \leq 0.05$) among the ExpLines.

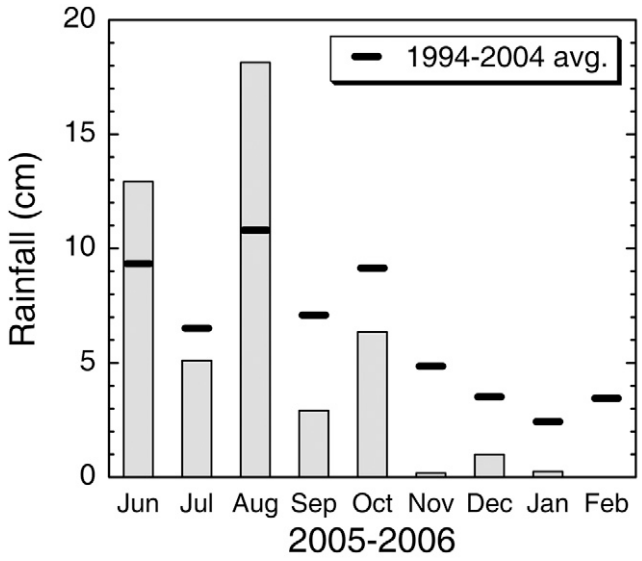

Figure 1. Monthly rainfall during the summer fallow and wheat forage crop cycle near the research plots of the breeding trial experiment located in Garfield County, Oklahoma.

Figures 3, 4, and 5 are histograms depicting the data distributions of tannin traits for the 221 ExpLines evaluated in the BT Exp. Most of the data exhibited either Normal (Shapiro-Wilk W Test, $P > 0.05$) or LogNormal (Kolmogorov's D, $P > 0.05$) distributions (statistical tests not shown). However, extractable tannic acid–eq TP and epicatechin-eq CT concentrations of the late fall forage sample and the quebracho-eq CT concentration of the regrowth sample failed the goodness-of-fit test for the Normal and LogNormal distributions.

Maximum forage concentrations of extractable tannic acid equivalent in the ExpLines were about 2.5-fold greater than minimum concentrations (Fig. 3). Among the ExpLines, significant differences ($P \leq 0.05$) in tannic acid–eq TP concentration were found for the late fall and winter samples but not the winter regrowth sample. Among the four Check varieties, forage of Jagalene consistently had less ($P \leq 0.05$) extractable tannic acid–eq TP concentrations than Duster for each forage sample collection. The concentration of total phenolics averaged across the four Check varieties was more in late fall forage than that of regrowth forage, which was greater than that of late winter forage (paired t tests, $P \leq 0.05$).

Maximum forage concentrations of quebracho-eq CT were 4.6- to 5.7-fold greater than minimum concentrations (Fig. 4). Only the late-winter-collected forage was found to have significant differences ($P \leq 0.05$) in quebracho-eq CT concentrations among the ExpLines. Among the four Check varieties, no significant differences were found in quebracho-eq CT concentrations in late fall forage samples. In contrast, concentrations of quebracho-eq CT in regrowth and late winter forage samples of Overley were usually greater ($P \leq 0.05$) than the concentrations of other Check varieties and the overall mean of the

ExpLines. Quebracho-equivalent condensed tannin concentration averaged across the four Check varieties was less in late fall forage than that of late winter forage, which was less than that of late winter regrowth forage (paired *t* tests, $P \leq 0.05$).

Table 2. The ANOVA results for the augmented, randomized, complete block design breeding trial experiment (BT Exp) located in Garfield County, Oklahoma. The ANOVA model provides significant effects test of Checks (the four varieties Duster, Endurance, Jagger, and Overley) and of 221 experimental lines (ExpLines) nested within Checks, with each Check variety contained in 12 blocks of the BT Exp.

Forage response variable†	Sample‡	Source of variation	
		Checks	ExpLines
		——— $P > F$ ———	
Tannic acid–eq TP	Late fall	0.0111	<0.0001
	Late winter RG	0.0170	0.0653
	Late winter	<0.0001	0.0001
Quebracho-eq CT	Fall	0.5469	0.7408
	Late winter RG	0.0002	0.7525
	Late winter	0.0001	0.0039
Epicatechin-eq CT	Fall	0.3235	0.0010
	Late winter RG	0.5527	0.2687
	Late winter	0.1463	0.8618

†Tannic acid–eq TP, tannic acid–equivalent total phenolics; quebracho-eq CT, quebracho-equivalent condensed tannin; epicatechin-eq CT, epicatechin-equivalent condensed tannin.

‡Forage samples were collected at the onset of the beginning of grazing in late fall and again in late winter before jointing for regrowth from the late fall sample (Late winter RG) and a previously unclipped sample (Late winter).

Excluding two low outlier forage concentrations of epicatechin-eq CT for the late fall sample among the ExpLines, maximum concentrations of epicatechin-eq CT were about threefold greater than minimum concentrations for all sample collections (Fig. 5). Among the ExpLines, only the late-fall-collected forage sample was found to have significant differences ($P \leq 0.05$) in the epicatechin-eq CT concentrations. Within each sample collection, forage epicatechin-eq CT concentrations were not significantly different ($P > 0.05$) among the Check varieties and overall mean of the ExpLines. Epicatechin-equivalent condensed tannin concentrations, however, were slightly less in forage from late fall samples than the regrowth and late winter samples (paired *t* tests, $P \leq 0.05$).

DISCUSSION

Plants produce a complex and diverse array of secondary metabolites that have a wide range of essential functions for growth and survival. Among the many secondary metabolites, tannins are a group of polyphenolic compounds that differ markedly in chemical structure and biological activity (Mueller-Harvey, 2006) but share a similarity in their binding of proteins in aqueous solution, thereby affecting the nutritive value of forage. Tannins may be divided into two groups: (i) hydrolyzable tannins (derivatives of galloyl glucoses; e.g., tannic acid) and (ii) condensed tannins (polymers of flavan-3-ols). The level of tannins in plants can vary among species and genotype (Roberts et al., 1993; Springer et al., 2002) and can change due to biotic stress

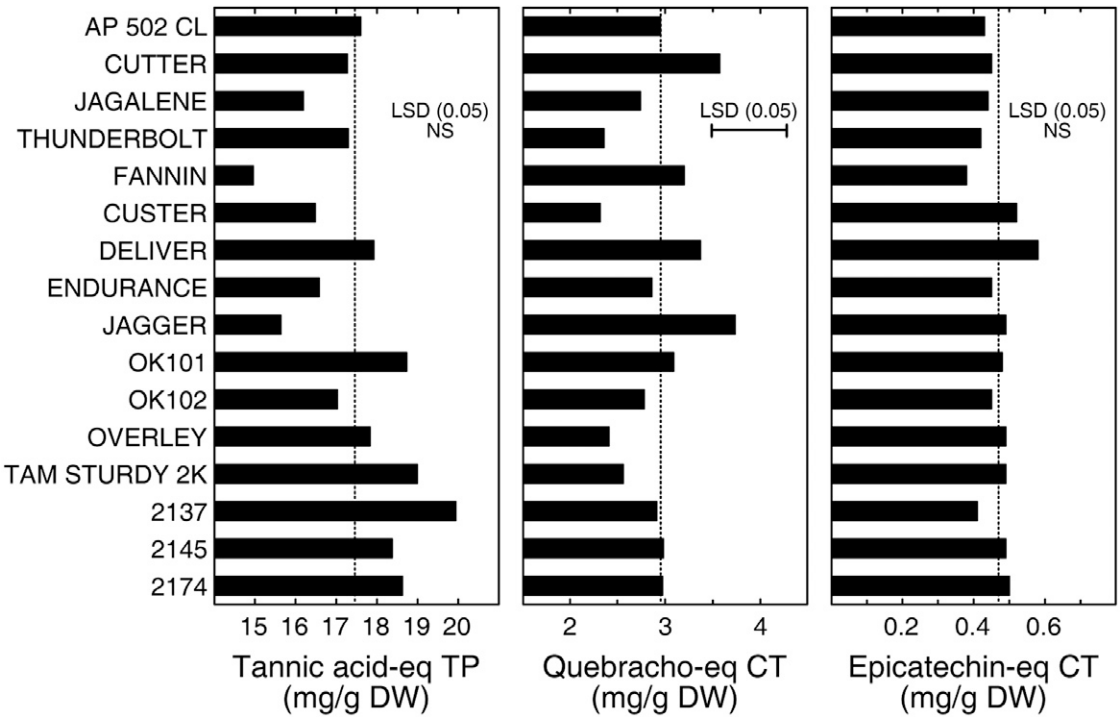

Figure 2. Methanol–chloroform–water extractable measurements of phenolics measured as tannic acid–equivalent total phenolics (tannic acid–eq TP, Prussian blue assay), quebracho-equivalent condensed tannins (quebracho-eq CT, acid-butanol assay), and epicatechin-equivalent condensed tannins (epicatechin-eq CT, vanillin assay) for wheat varieties grown in Canadian County and sampled 10 Dec. 2004. Vertical black dashed line in each panel marks the overall mean.

(herbivory and disease; Dixon and Paiva, 1995; Karban and Baldwin, 1997; Richard et al., 2000) and environmental factors (light, nutrients, water, and temperature; Barry and Forss, 1983; Anuraga et al., 1993; Dudt and Shure, 1994; Hemming and Lindroth, 1999). The order of importance of these factors is not consistent. For example, fertilization and shade have a greater influence on tannins than genotype or foliar damage in *Betula pubescens* ssp. *tortuosa* (Ruohomäki et al., 1996), while in *Betula pendula*, the genotype is the most important, followed by shading and damage (Keinanen et al., 1999). Among the environmental factors, the effect of shading (low light intensity) on decreased tannin concentration is least ambiguous. The levels of light and sucrose, a principal product of photosynthetic-generated triose phosphates, are involved in the signal transduction pathway affecting expression of genes, resulting in a strong up-regulation of the flavonoid and anthocyanidin biosynthetic pathways of model plants (Paolocci et al., 2005; Solfanelli et al., 2006).

Solar radiation measured a few days before and during the forage sample collection periods for the VT Exp (Table 1) exceeded 71% of the daily solar radiation (overall avg. 86%) and was in excess of 56% of the daily solar radiation for the BT Exp (avg. 73% late November sample and 84% late February sample). These conditions should favor formation of phenolic compounds and tannins (Dudt and Shure, 1994; Hemming and Lindroth, 1999). Fall precipitation for the VT Exp was more than adequate (47% above normal) and would not have been a limiting factor for growth of wheat forage. The low amounts of precipitation received after planting for the BT Exp in 2005 (Fig. 1) might be expected to decrease dry matter accumulation and perhaps favor elevated tannin concentrations as a consequence of less dilution due to dry matter accumulation (Anuraga et al., 1993). Because of the absence of extremely

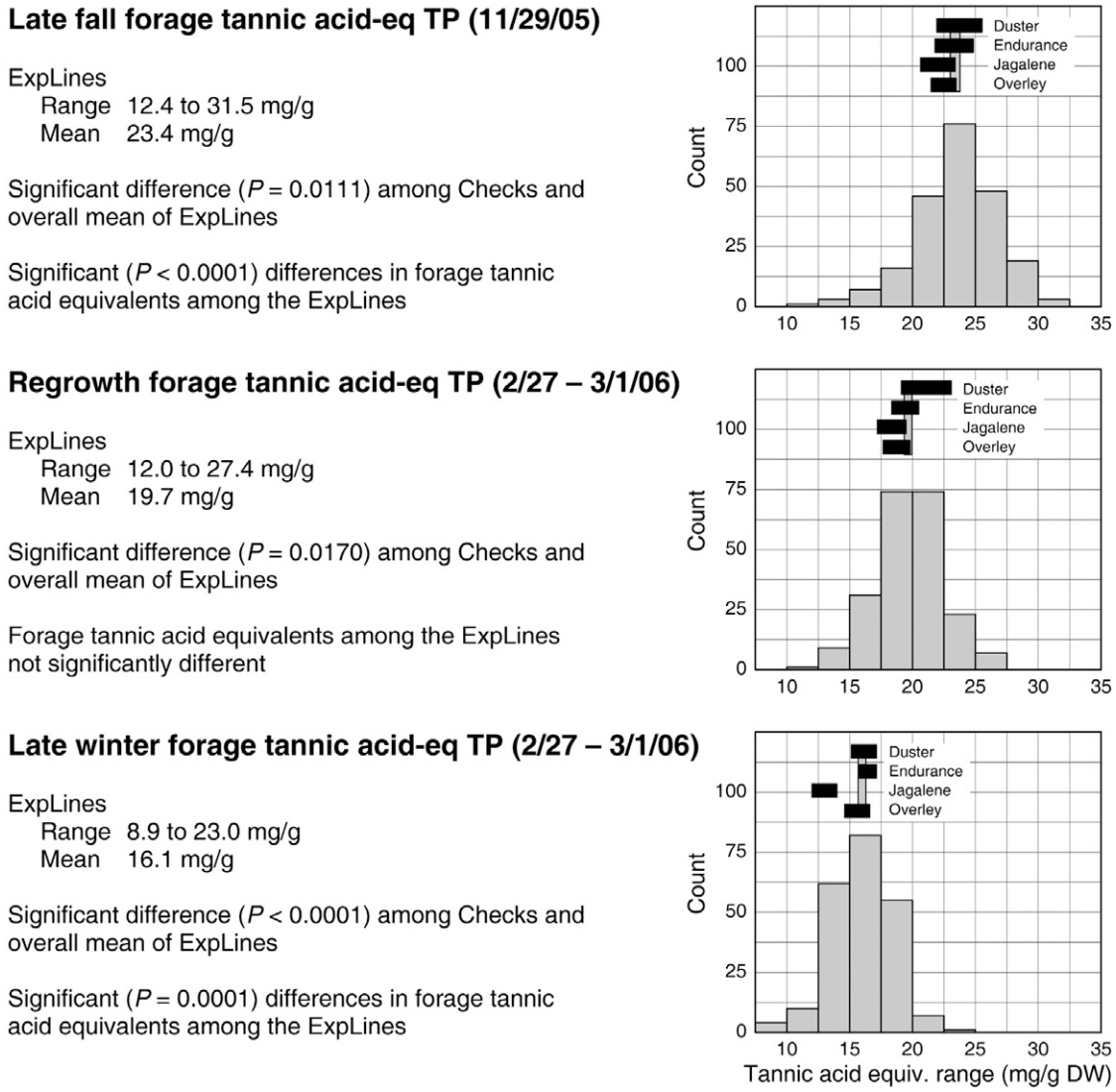

Figure 3. Tannic acid–equivalent total phenolics (tannic acid–eq TP) distributions for forage sampled from the experimental lines (ExpLines) at the onset of the beginning of grazing in late fall and in late winter pre–Feekes Growth Stage 6. Black horizontal bars overlapping the small gray vertical bar represent lower and upper 95% confidence intervals of the least-squares means of the Checks and ExpLines, respectively.

adverse climate conditions, we suspect that the levels of total phenolics and condensed tannin assay reactive substances that we measured in wheat at the onset of late fall grazing and end of tiller formation would be within a "normal" range of levels expected for the vegetative phase of winter wheat forage grown in Oklahoma.

Wheat Forage Total Phenolics and Condensed Tannins

Variety Trial Experiment

Total phenolics expressed as tannic acid equivalents of the 16 wheat varieties evaluated in the VT Exp ranged from 15 to 20 mg g^{-1} dry wt., but the observed variation was not significant. Although quebracho-eq CT were significantly different among the 16 varieties, differences in epicatechin-eq CT were not significant (Fig. 2), and these two functional group assays for condensed tannin were not correlated ($r = 0.18$, $P = 0.156$).

For our condensed tannin assays, we chose two methods that are specific for condensed tannins and compounds containing *meta*-diphenol structures. While both assays suffer from several problems (Schofield et al., 2001), they still offer an opportunity to evaluate condensed tannin concentrations. We used standards that were not derived from wheat materials. Consequently, while our results are not quantitative for condensed tannins specific to wheat forage, they do provide us a measure of differences in relative abundance of compounds that have reactivity equivalent to our external standards. The acid-butanol assay is a colorimetric assay that involves the acid-catalyzed oxidative depolymerization of condensed tannins to yield red-colored anthocyanidin monomers. The vanillin assay detects the red color produced by

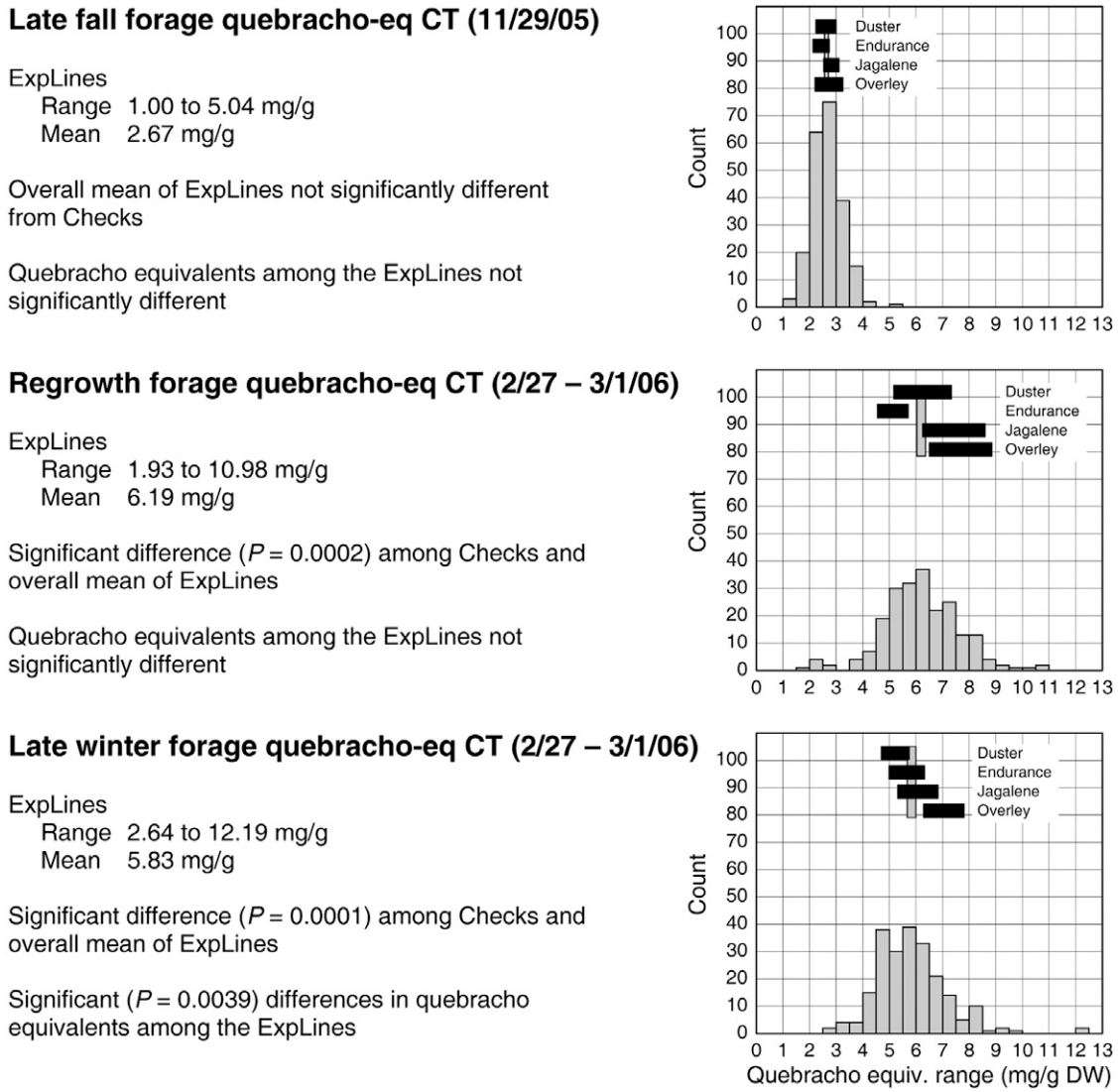

Figure 4. Quebracho-equivalent condensed tannins (quebracho-eq CT) distributions for forage sampled from the experimental lines (ExpLines) at the onset of the beginning of grazing in late fall and in late winter pre–Feekes Growth Stage 6. Black horizontal bars overlapping the small gray vertical bar represent lower and upper 95% confidence intervals of the least-squares means of the Checks and ExpLines, respectively.

the reaction of vanillin with appropriately substituted flavanols. For our acid-butanol assay, the relationship between the epicatechin standard (0.25–2.25 mg) and quebracho-eq CT was

$$\text{mg of quebracho-eq CT} = -0.0871 + 0.584 \times (\text{mg of epicatechin})$$

$$R^2 = 0.995,\ P < 0.0001$$

and for the vanillin assay, the relationship between the quebracho standard (0.0025–0.0375 mg) and the epicatechin standard was

$$\text{mg of epicatechin-eq CT} = 0.144 + 0.136 \times (\text{mg of quebracho})$$

$$R^2 = 0.999,\ P < 0.0001$$

For the acid-butanol assay, the terminal flavanol unit is not converted to anthocyanidin and does not contribute to the red color of the assay, and with the vanillin assay, only some of the internal flavanol units in a condensed tannin will react with vanillin and contribute to the formation of the assay red color. Consequently, if condensed tannins in wheat forage have a range in the degree of polymerization as is observed in legumes (McAllister et al., 2005), the two assays would not necessarily be correlated for the extracts, in contrast to the external standards chosen for the assays.

While it is likely that the adapted wheat varieties we evaluated contain condensed tannins, the concentration of these compounds noted for their protein precipitation capacity are substantially less (>seven-fold) than the 20 to 40 g kg^{-1} dry wt. concentrations of condensed tannins proposed as a level sufficient to prevent bloat (Barry et al., 1986; Aerts et al., 1999). An abundant concentration of tannic acid–eq TP was detected. Some compounds in the total phenolic acid mixture would be expected to

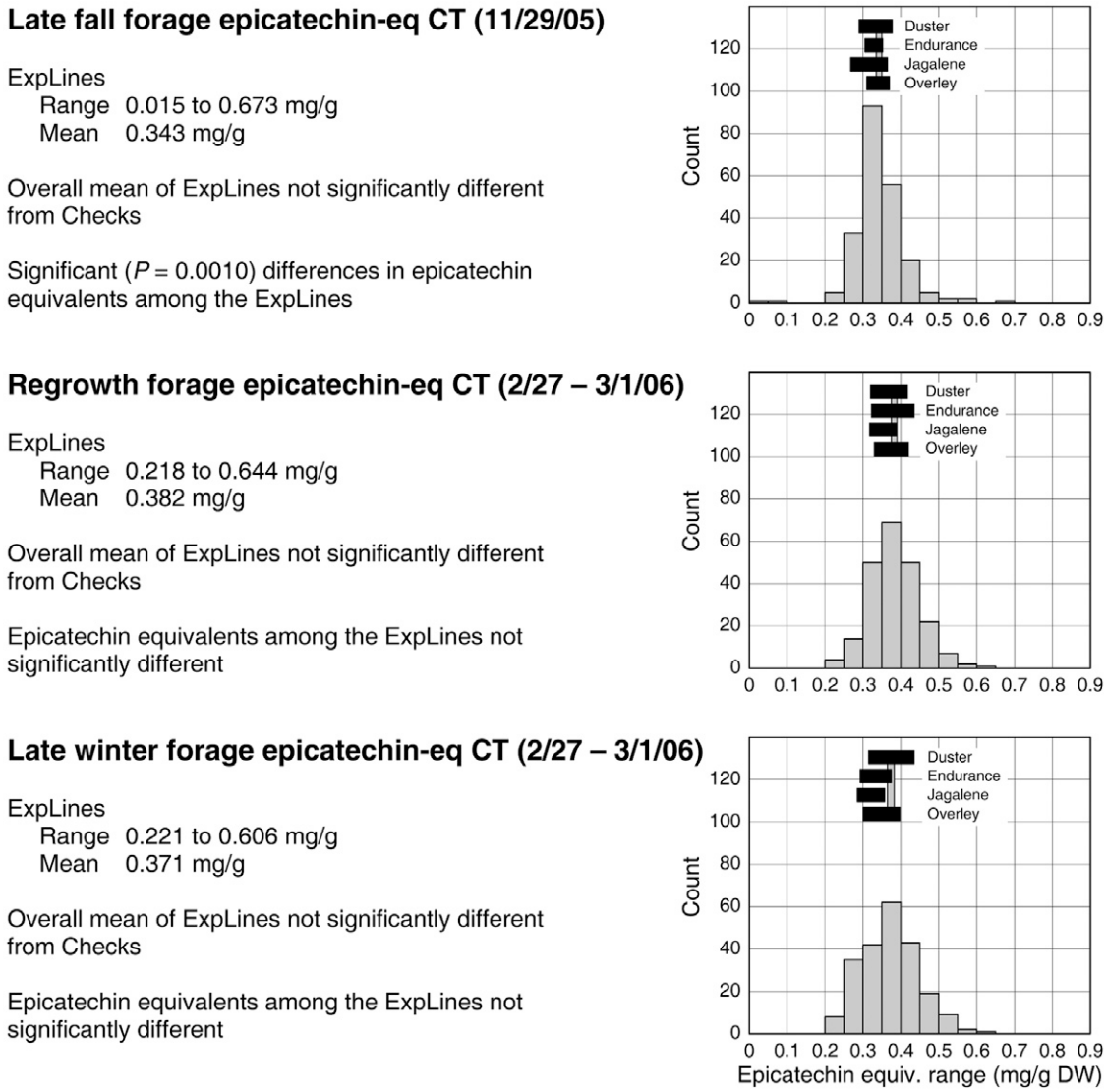

Figure 5. Epicatechin-equivalent condensed tannins (epicatechin-eq CT) distributions for forage sampled from the experimental lines (ExpLines) at the onset of the beginning of grazing in late fall and in late winter pre–Feekes Growth Stage 6. Black horizontal bars overlapping the small gray vertical bar represent lower and upper 95% confidence intervals of the least-squares means of the Checks and ExpLines, respectively.

have protein precipitation properties that may lessen the formation of stable foams in the rumen. Condensed tannins are recognized as potent precipitators of protein, and the degree of condensed tannin polymerization is directly proportional to their capacity to precipitate protein (Horigome et al., 1988). With our forage extracts we did not detect protein precipitation following a semi-quantitative protocol (Hagerman, 1987) that uses bovine serum albumin (BSA) in agarose plates (data not shown). The choice of protein in precipitation assays can give markedly different results. Ribulose 1,5-bisphophate carboxylase EC 4.1.1.39, the primary soluble protein in forage consumed by grazing livestock, is more readily precipitated by condensed tannins than BSA (Martin and Martin, 1983; McAllister et al., 2005) and may be more suitable for testing wheat forages for protein precipitation properties and warrants further study.

Breeding Trial Experiment

Concentrations of extractable tannic acid–eq TP (Fig. 3), quebracho-eq CT (Fig. 4), and epicatechin-eq CT (Fig. 5) of the Check varieties and means of the ExpLines harvested in the late fall were similar to the mean concentrations of these phenolics in the early winter forage of 16 varieties grown the year before in the VT Exp. Some of the experimental entries had concentrations that were substantially greater than the Checks, but even those entries in the uppermost concentration ranges of phenolic compounds that were detected by the condensed tannin assays would be two- to fourfold less than levels believed sufficient to prevent bloat (Barry et al., 1986; Aerts et al., 1999). Similar to the VT Exp forage samples, a test of protein precipitation (Hagerman, 1987) of forage extracts from several of the ExpLines with uppermost concentrations of tannic acid-eq TP were negative (data not shown). Nevertheless, controlled matings using lines exhibiting elevated levels of tannins may provide additional wheat lines with even greater concentrations of tannins.

Levels of extractable total phenolics of winter regrowth and forage sampled only once in late winter were less than those in the late fall forage sampled at the onset of the grazing cycle. Environmental stresses that stimulate the shikimic acid pathway and reduce incorporation of shikimate-derived amino acids into proteins (protein competition model; Jones and Hartley, 1999) should increase the amount of precursors available for secondary metabolite and tannin synthesis. Despite the lack of rainfall for the BT Exp located in Garfield County (Fig. 1) that likely resulted in reduced forage production linked to water stress, concentrations of extractable total phenolics decreased 16% in the winter regrowth forage samples and 31% in late winter forage samples. In contrast, the concentration of quebracho-eq CT increased (Fig. 4), while concentration of epicatechin-eq CT (Fig. 5) were similar for all samples. There is no clear explanation to reconcile these effects on the different phenolic fractions.

Experimental lines with abundant extractable phenolics may offer other benefits that should not be ignored (Wu et al., 2001a; Bertin et al., 2003). Many phenolic compounds inhibit seed germination and plant growth (Rice, 1984). The predominant phenolic acids with allelopathic activity include *p*-hydroxybenzoic, vanillic, *p*-coumaric, syringic, and ferulic acids (Guenzi and McCalla, 1996; Blum et al., 1991). These allelopathic phenolic acids in 58 accessions of 17-d-old wheat seedlings ranged from about 0.09 to 0.45 g kg^{-1} dry wt. of shoot and 0.09 to 0.55 g kg^{-1} dry wt. of root tissues (Wu et al., 2000a; Wu et al., 2001a). Besides the effect of wheat residues on germination and growth of weeds, seedlings with elevated levels of allelopathic phenolic acids that release these compounds from their roots can have a competitive advantage against the growth of annual grasses (Spruell, 1984; Wu et al., 2000b, 2001b).

Negative correlations between forage yields (data not shown) and tannic acid–eq TP concentrations ranged from −0.36 to −0.43 ($P < 0.0001$), which would likely be the consequence of dilution of the extractable phenolics by dry matter accumulation. For the condensed tannin concentrations, correlations with forage yields were also negative but less strong (data not shown). Correlations of fall tannic acid–eq TP concentrations were weak for regrowth (0.21, $P < 0.001$) and moderate (0.45, $P < 0.0001$) for late winter concentrations of tannic acid–eq TP, while those for quebracho-eq CT concentrations were weak (0.16, $P = 0.008$) and for concentrations of epicatechin-eq CT, nonsignificant ($P > 0.05$). The absence of strong correlations of these classes of phenolic compounds across forage harvests indicates that the concentrations of total phenolics and condensed tannins of the entries across harvests were not predictable and were likely variable for an entry.

CONCLUSIONS

This research provides evidence for the presence of extractable compounds that have positive reactions in two different assays that are commonly used to measure condensed tannins. Undoubtedly there are marked differences in total phenolic and condensed tannin substances among this genetically diverse set of ExpLines, representing levels both less than and greater than those in released varieties. While detection of ExpLines with elevated concentrations of tannin-like reactive substances is encouraging, the levels measured must be evaluated against their effectiveness of providing an alternative choice to reduce or prevent occurrence of wheat pasture bloat. The concentrations of these compounds, however, are low compared to levels probably required to offer a forage-derived defense against pasture bloat. A lower limit of condensed tannin for bloat-safe forage is likely in excess of 20 g kg^{-1} dry wt., which

is much greater than even the highest level we detected in the ExpLines. Perhaps those lines with the most abundant levels could be used in a concerted breeding approach to increase the level of tannins in wheat forage. Because total phenolic and condensed tannin concentrations were not selected traits in this study, a genetic heritability trial would provide evidence that a conventional breeding approach has merit. Also, the occurrence of tannins in wild relatives of wheat should be evaluated as a source of genes for use in a breeding program.

Acknowledgments

Assistance with collection, processing, and analysis of forage samples was provided by Jeff Weik, Kolby Bollinger, Kory Bollinger, and Clendon Tucker of the USDA-ARS Grazinglands Research Laboratory. Wayne Whitmore and other Oklahoma State University personnel established the experimental plots. Partial financial support from the Oklahoma Wheat Research Foundation and the Oklahoma Wheat Commission made this research possible. Oklahoma Mesonet data were provided courtesy of the Oklahoma Mesonet, a cooperative venture between Oklahoma State University and the University of Oklahoma and supported by the taxpayers of Oklahoma.

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